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XRCC3 ATPase Activity Is Required for Normal XRCC3-Rad51C Complex Dynamics and Homologous Recombination*

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Homologous recombinational repair preserves chromosomal integrity by removing double-strand breaks, cross-links, and other DNA damage. In eukaryotic cells, the Rad51 paralogs (XRCC2/3, Rad51B/C/D) are involved in this process, although their exact functions are largely undetermined. All five paralogs contain ATPase motifs, and XRCC3 exists in a single complex with Rad51C. To examine the function of this Rad51C-XRCC3 complex, we generated mammalian expression vectors that produce human wild-type XRCC3 or mutant XRCC3 with either a nonconservative mutation (K113A) or a conservative mutation (K113R) in the GKT Walker A box of the ATPase motif. The three vectors were independently transfected into Xrcc3-deficient irs1SF Chinese hamster ovary cells. Wild-type XRCC3 complemented irs1SF cells, albeit to varying degrees, whereas ATPase mutants had no complementing activity, even when the mutant protein was expressed at comparable levels to that in wild-type-complemented clones. Because of dysfunction of the mutants, we propose that ATP binding and hydrolyzing activities of XRCC3 are essential. We tested *in vitro* complex formation by wild-type and mutant XRCC3 with His_6-tagged Rad51C upon co-expression in bacteria, nickel-affinity purification, and Western blotting. Wild-type and K113A mutant XRCC3 formed stable complexes with Rad51C and co-purified with Rad51C, whereas the K113R mutant did not and was predominantly insoluble. The addition of 5 mM ATP but not ADP also abolished complex formation by the wild-type proteins. These results suggest that XRCC3 probably regulates the dissociation and formation of Rad51C-XRCC3 complex through ATP binding and hydrolysis with both processes being essential for the ability of the complex to participate in homologous recombinational repair.

Homologous recombinational repair (HRR)[1] is a major DNA repair pathway that preserves chromosomal integrity during DNA replication and contributes to the removal of double-strand breaks and interstrand cross-links from exogenous agents (see reviews in Refs. 1–3). HRR probably helps prevent double-strand breaks from arising during normal DNA replication and promotes their removal in an error-free manner when they do arise. In eukaryotic cells, this process is mediated by the highly conserved Rad51 DNA strand transferase and associated proteins that include distant relatives of Rad51, which are referred to as the Rad51 paralogs (XRCC2, XRCC3, Rad51B, Rad51C, and Rad51D). At least two stable complexes (a dimeric complex composed of XRCC3 and Rad51C and a larger complex composed of XRCC2, Rad51B, Rad51C, and Rad51D) have been found (4–8). However, the exact role(s) these complexes play in HRR remains unclear. Mutations in the paralog genes lead to excessive spontaneous chromosomal aberrations and sensitivity to ionizing radiation (IR) and DNA cross-links (9–13).

Rad51 contains ATPase motifs composed of the Walker A and B boxes, and its DNA binding (14) and strand transferase activities (15, 16) are ATP-dependent. Structural analysis of archeal Rad51 shows that the ATPase binding site is composed of Walker A and Walker B motifs from different Rad51 monomers (17), suggesting that ATP binding may act as a "bridge" for protein-protein interaction. Interestingly, a conservative lysine to arginine mutation in the Walker A box resulted in a mutant human Rad51 with efficient DNA binding and strand transferase activity while a nonconservative lysine to alanine mutation inactivated Rad51 (18). Because conservative mutation at Walker A motifs is thought to abrogate ATP hydrolysis without inhibiting ATP binding and because nonconservative mutations result in a nonbinding, nonhydrolyzing protein, these results indicate that ATP binding alone may be sufficient to maintain Rad51 function.

The Rad51 paralogs also contain Walker A and B ATPase motifs. However, unlike Rad51, the biological significance of these ATPase motifs is not clearly established. In *Saccharomyces cerevisiae*, the Rad51 paralogs, Rad55 and Rad57, showed differential requirements for the Walker A motifs. In a complementation analysis of mutant lines, neither nonbinding nor nonhydrolyzing mutants of Rad55 were able to complement IR sensitivity, whereas both mutants in Rad57 showed partial complementation (19). This result could indicate that the ATPase binding site of yeast Rad51 paralogs may be composed of the Walker A box of Rad55 and the Walker B box of Rad57 and that ATPase binding sites may act as sites of protein-protein interaction for the paralogs, much like the sites of interaction in Rad51.

Genetic analysis of the human Rad51 paralogs, Rad51C and XRCC2, has been conducted by ectopic expression of the paralogs in Chinese hamster ovary (CHO) cell mutants. Interest-

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[1] The abbreviations used are: HRR, homologous recombinational repair; MMC, mitomycin C; CHO, Chinese hamster ovary; IR, ionizing radiation.

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ingly, a similar trend of differential requirement for the Walker A motif seem to be emerging for the human Rad51 paralogs as well. A XRCC2 mutant that is unable to bind and/or hydrolyze ATP showed partial complementation for the CHO mutation (20), whereas an analogous mutation in Rad51C showed no complementing activity (21). When Rad51C retained its ability to bind ATP, it was able to partially restore MMC resistance in irs3 mutant V79 cells, suggesting that ATP binding, rather than hydrolysis, may be critical for Rad51C function. Rad51B also hydrolyzes ATP, and the Rad51C-Rad51B dimer exhibited additive ATPase activity, although the biological significance of the Rad51B ATPase activity is unknown (5, 22).

We investigated whether the ATPase activity of XRCC3 is required for homologous recombinational repair (23, 24) as measured by its ability to complement the CHO irs1SF cell line (9, 25, 26). We report that the integrity of the Walker A motif of XRCC3 is required for biological activity and governs Rad51C-XRCC3 complex formation. This study is the first to address the relationship between complex formation and biological activity of the Rad51 paralogs.

EXPERIMENTAL PROCEDURES

Generation of Mammalian XRCC3 Expression Constructs—Wild-type *XRCC3* cDNA was amplified by PCR from IMAGE clone 3139703 and inserted into the pEF6-V5/His vector by TOPO-cloning (Invitrogen). The orientation was confirmed by restriction digestion and direct sequencing of the entire cDNA. Lys to Arg and Lys to Arg mutations were introduced at Lys-113 using the QuikChange site-directed mutagenesis kit (Stratagene). The missense mutations were also confirmed by direct sequencing.

Cell Culture and Generation of XRCC3 Expressing irs1SF Cells—Cells were grown in monolayer or suspension culture in α-minimal essential medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cell lines derived from irs1SF, which stably express wild-type XRCC3 or the ATPase mutants, were created by transfection of the XRCC3 expression constructs and subsequent selection for the drug resistance marker. 2×10^7 irs1SF cells were washed and resuspended in 1 ml of cold electroporation buffer (20 mM HEPES, pH 7, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose), mixed with 10 μg plasmid DNA, electroporated at 250 V/1600 microfarads, and plated in a T150 flask for 24 h to allow for expression of the blasticidin resistance gene. Cells were then plated into 10-cm dishes at $\sim 5 \times 10^5$ cell/dish in 20 ml of medium containing 5 μg/ml blasticidin (Invitrogen) and incubated at 5% CO_2 and 37 °C for 12 days when most colonies were clearly visible by eye. Twelve individual colonies were isolated and expanded as clones for each transfection.

Western blotting was performed with nuclear and cytoplasmic extracts of each clone to confirm that XRCC3 was being expressed. Extracts were prepared from cells using the NE-PER nuclear and cytoplasmic extraction kit (Pierce), quantitated by the Bradford method, and normalized for equal loading. Extracts were separated on a 12% PAGE gel and transferred to Hybond-ECL membrane (Amersham Biosciences) according to standard protocol. Filters were probed with 1:500 diluted primary anti-XRCC3 antibody (Novagen) and visualized by ECL detection. The film was then scanned, and band strength was quantitated using ImageQuant (Amersham Biosciences). Gels were loaded at a minimal level of detection to stay within the linear range of the film.

MMC and IR Complementation Analysis—Mutagen sensitivities were determined by colony formation in 10-cm dishes. For each dose, 300 cells were plated in triplicate and allowed 12 days of growth. Dishes were then rinsed with saline, fixed with 95% ethanol, and stained with Gram crystal violet (BD Dickinson). Exposure to MMC was conducted in 10-ml suspension cultures. At each dose, 1×10^5 cells were exposed to MMC for 60 min at 37 °C, centrifuged, and resuspended in fresh medium for plating. For exposure to IR, ^{137}Cs γ-irradiation was performed on suspension cultures (1×10^4 cells/ml) in 15-ml polypropylene tubes on ice.

Purification and Detection of Rad51C-XRCC3 Heterodimer—To co-express Rad51C and XRCC3 in *E. coli*, we replaced the origin of replication in pET15b (Novagen) from the colE1 origin with the p15a origin from pACYC177 (New England Biolabs). Using the modified pET15b, we generated a vector for Rad51C with an N-terminal His_6 tag. We also removed all of the tag sequences from pET29b (Novagen) to create a vector for untagged XRCC3. BL21 DE3 (Novagen) was co-transformed with vectors for His-tagged Rad51C and untagged XRCC3 and selected for resistance to both ampicillin and kanamycin.

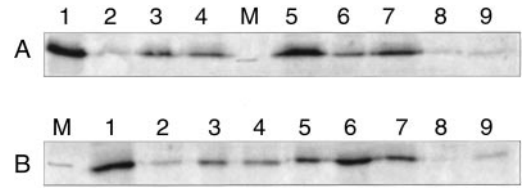

FIG. 1. **Western blotting for XRCC3 in irs1SF transfectants.** *A*, cytoplasmic fractions. *B*, nuclear fractions. *M*, marker (38 kDa). *Lane 1*, 1SFK3; *lane 2*, 1SFK7; *lane 3*, 1SFK8; *lane 4*, 1SFK12; *lane 5*, 1SFR3; *lane 6*, 1SFR7; *lane 7*, 1SFR11; *lane 8*, 1SFA9; and *lane 9*, 1SFA10. Clones having the normal Walker A box are designated 1SF**K**, and mutant clones designated 1SF**R** and 1SF**A** have Lys to Arg or Lys to Ala substitutions, respectively. Quantitation was conducted on these results and other films of similar band intensity. Two independent experiments were conducted to verify reproducibility by obtaining relative signal ratios for 1SFK12 and 1SFKA9. The ratios were 4.1 and 4.2 in the two experiments.

Liquid cultures were inoculated from individual clones, and recombinant protein expression was induced at $A_{600} = 0.5$ with 1 mM isopropyl-β-D-thiogalactopyranoside for at least 16 h at 16 °C. Cells were lysed in the same buffer as the binding buffer for purification (50 mM sodium phosphate, 5 mM Tris, pH 8.5) by freeze-thaw lysis followed by sonication on ice. Lysates were centrifuged ($15{,}000 \times g$) at 4 °C, and the supernatant was loaded on to nickel-charged HiTrap chelating HP using the ÄKTA fast protein liquid chromatography (Amersham Biosciences). The loaded column was washed with 50 mM sodium phosphate, 5 mM Tris, 5 mM imidazole, and 10 mM imidazole before eluting on a gradient with 50 mM sodium phosphate, 5 mM Tris, and 250 mM imidazole. One elution peak containing the dimer fraction was seen in all of the purification experiments. For each dimer pair, at least two independent transformations were conducted to ensure reproducibility. When purification containing 5 mM ATP or 5 mM ADP was conducted, induced cultures were split into two aliquots. One aliquot was used in the purification with ATP or ADP in the lysis/binding buffers, and the second aliquot was purified with regular lysis/binding buffer. Fractions corresponding to the peaks detected were separated on a 12% PAGE gel and probed with either anti-XRCC3 antibody as described above or with anti-Rad51C antibody diluted at 1:1000 (Novagen). The remaining steps for the detection of Rad51C were identical to XRCC3 detection.

RESULTS

Expression Level of Wild-type Human XRCC3 Determines Its Ability to Complement MMC and IR Sensitivity of irs1SF—We isolated 12 independent wild-type XRCC3-expressing transfectants derived from the CHO mutant line irs1SF by screening blasticidin-resistant colonies. These clones varied in the level of XRCC3 protein as illustrated in Fig. 1 for four of them, and the extent of complementation for MMC resistance ranged from 1 to 27% (Fig. 2*A* and Table I). We found that a narrow range of XRCC3 protein levels was associated with efficient complementation of MMC and IR sensitivity in irs1SF (Table I). At very high or very low levels, XRCC3 did not complement MMC sensitivity. Because of interspecies differences and antibody specificity for human XRCC3, we are unable to compare the XRCC3 ectopic level with that of wild-type Xrcc3 in parental AA8 cells. Therefore, we analyzed the XRCC3 level in all of the clones relative to those in 1SFA9 clone, which was our lowest detectable expression level.

We conducted complementation analysis for IR sensitivity only on certain clones. 1SFK8 is noteworthy in having substantial complementation with both MMC (20%) and IR (60%), which is higher than we found previously for a smaller set of transfectants (26). This differential effect in favor of higher correction for IR sensitivity was seen previously (26). Clone 1SFK6 was also well corrected (26%) for MMC but was less well corrected for IR and had a weakly adherent colony morphology.

Both Conservative and Nonconservative ATPase Mutations in the Walker A Box Abrogate XRCC3 Function—When either

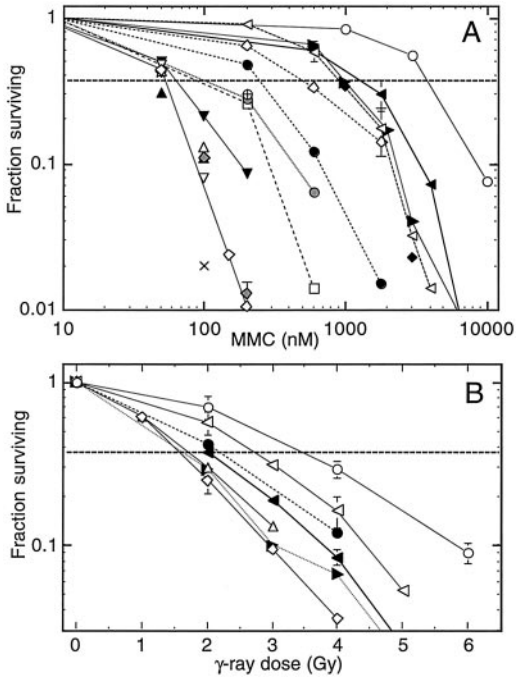

FIG. 2. **Survival curves for colony forming ability of cells exposed to MMC or IR**. *A*, exposure to MMC for 1 h. Symbols: AA8 (○); irs1SF (◇); 1SFK1 (⊕); 1SFK2 (⊙); 1SFK3 (●); 1SFK6 (◀); 1SFK7 (□); 1SFK8 (◁); 1SFK9A (◇); 1SFK12 (▶); 1SFK12A (◆); 1SFR3 (△); 1SFR7 (×); 1SFR10 (◈); 1SFR11 (▲); 1SFA9 (▽); and 1SFA10 (▼). *B*, exposure to ^{137}Cs γ-rays. Symbols are the same as in *panel A*. 1SFR10, which had no recombinant protein expression, is included as a transfection control. Transfectants 1SFK9A and 1SFK12A were selected directly in MMC instead of blasticidin.

TABLE I
XRCC3 expression levels and degree of complementation of MMC sensitivity or irs1SF cells

NA, not applicable.

Cell line	D_{37} for MMC	Fractional correction	Relative protein level
	nM		
AA8	3800	1.0	NA
irs1SF	50	0.0	NA
1SF**K**7	90	0.01	1.2
1SF**K**6	1020	0.26	2
1SF**K**12	980	0.1	4
1SF**K**8	900	0.20	5
1SF**K**3	230	0.04	14
1SF**K**2	90	0.01	17
1SF**K**1	99	0.01	31
1SF**R**3	50	0.0	6
1SF**R**11	62	0.0	7
1SF**R**7	50	0.0	11
1SF**A**9	50	0.0	1
1SF**A**10	50	0.0	2

the conservative ATP binding mutation (K113R) or the nonconservative mutation (ATP-nonbinding, -nonhydrolyzing K113A) was expressed in irs1SF, no complementation was observed in any transfectants that expressed mutant protein. Of 12 blasticidin-resistant clones in each group, only three clones expressed K113R XRCC3 and two clones expressed K113A. Both clones expressing the K113A mutant had very little XRCC3, whereas K113R expression levels were substantial. None of these six clones had any complementation for MMC sensitivity (Fig. 2). To confirm that this loss of function was not caused by insufficient expression of the mutant protein, we compared the expression levels of mutant and wild-type XRCC3. For example, clone 1SFR3 had a level of the mutant protein comparable to 1SFK8 (20% correction) and 1SFA10 has more protein than

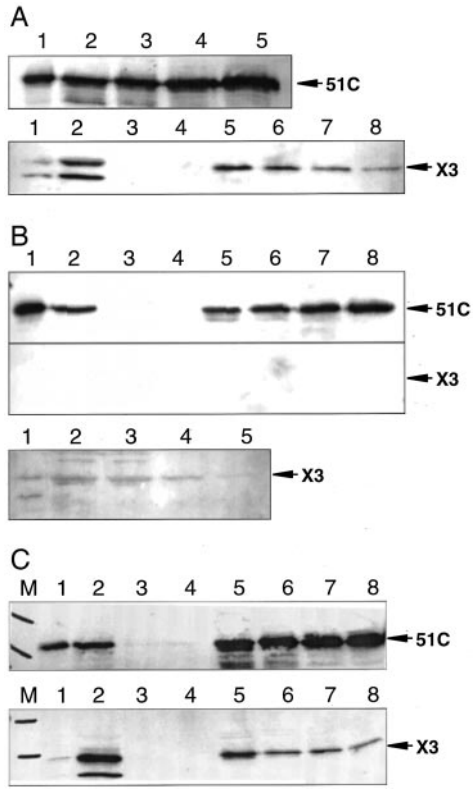

FIG. 3. **Complex formation by Rad51C with wild-type and mutant XRCC3**. *A*, wild-type XRCC3 is brought down by His-tagged Rad51C. *Top panel*: *lane 1*, cell lysate; *lanes 2–5*, eluted fractions. *Bottom panel*: *lane 1*, cell lysate; *lane 2*, flow-through; *lane 3*, first wash; *lane 4*, second wash; *lanes 5–8*, eluted fractions corresponding to *lanes 2–5* on *top panel*. *B*, K113R XRCC3 is not brought down by His-tagged Rad51C. *Top* and *middle panels*: *lane 1*, cell lysate; *lane 2*, flow-through; *lane 3*, first wash; *lane 4*, second wash; *lanes 5–8*, eluted fractions. *Bottom panel*, overexposed (20 h) blot of XRCC3. *Lane 1*, cell lysate; *lanes 2–5*, eluted fractions. *C*, K113A XRCC3 forms a stable complex with His-tagged Rad51C. *Top* and *bottom panels*: *lane 1*, cell lysate; *lane 2*, flow-through; *lane 3*, first wash; *lane 4*, second wash; *lanes 5–8*, eluted fractions corresponding to *lanes 2–5* in *top panel* of *A*. *M*, marker.

1SFK7, which had approximately ~2-fold increased MMC resistance (1% correction, which is significantly above base line) (Fig. 1). Neither mutation reduced the ability of XRCC3 to localize to the nucleus as similar nuclear/cytoplasmic ratios were observed for wild-type XRCC3 and the two mutant forms (Fig. 1).

Because 1SFR3 expresses K113R at a level comparable to 1SFK8, our most complemented clone, we tested whether the 1SFR3 mutant could complement the IR sensitivity of irs1SF. No significant increase in IR resistance occurred (Fig. 2*B*).

Rad51C-XRCC3 Complex Formation Is Blocked by the K113R Mutation but Not by the K113A Mutation—Because XRCC3 forms a stable dimer *in vivo* with Rad51C, we examined the stability of K113R and K113A mutant complexes by co-expressing Rad51C and XRCC3 in bacterial hosts. An N-terminal His tag on Rad51C was used to pull down XRCC3 by nickel-affinity purification of bacterial extracts. Almost all of Rad51C was found complexed with XRCC3 when either wild-type or K113A protein was expressed, and no visible difference was seen in the ratio of Rad51C to XRCC3 between wild-type and mutant complexes (Fig. 3). Unexpectedly, when K113R was expressed, Rad51C alone was detected in the eluted fractions; no XRCC3 co-purified. There was a very small amount of XRCC3 in all of the Rad51C-containing fractions but most was in the insoluble pellet fraction. When photographic film was processed to significantly overexpose Rad51C, a very faint band

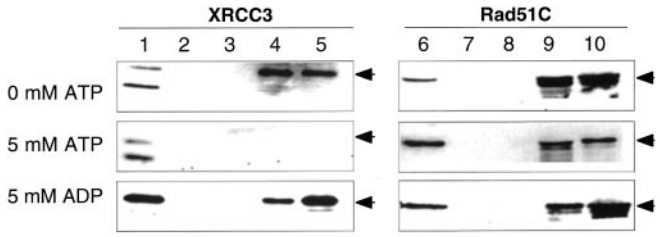

FIG. 4. **Complex formation by Rad51C with wild-type XRCC3 in the presence off excess ATP or ADP.** *Lanes 1–5* were probed for XRCC3, and *lanes 6–10* were probed for Rad51C. *Lanes 1* and *6*, flow-through; *lanes 2* and *7*, first wash; *lanes 3* and *8*, second wash; *lanes 4* and *9*, first eluted fraction; *lane 5*, 10-s eluted fraction. Purifications shown here in the *top* and *middle panels* (without or with ATP, respectively) were done from the same induced culture. The corresponding purification with regular lysis buffer for the culture used in the ADP purification was very similar to that shown here with ADP (data not shown). The *background lower band* seen in *lane 1* of the *top two panels* for XRCC3 was cut off in blot for the ADP purification.

was observed for XRCC3 in the eluted fractions. Starting with new transformants, we repeated this experiment three times and the results were the same.

Excess ATP Abolishes Rad51C-XRCC3 Dimer Formation—To confirm that XRCC3 ATPase activity was directly required for complex formation, we tested whether the presence of excess ATP during the purification process of wild-type XRCC3 and Rad51C would result in dimer dissociation. Because the K113R XRCC3 mutant should have maintained the ability to bind ATP, we mimicked the conditions of ATP being bound to the ATPase site of XRCC3 by having excess ATP in the lysis buffer during the purification step. We used the same induced culture for the purification with lysis buffer with 5 mM ATP or 5 mM ADP or no added nucleotide. We found that ATP, but not ADP, interfered with the co-purification of XRCC3 and Rad51C, similarly to what we saw for the K113R mutant (Fig. 4). Unlike with the purification with the K113R mutant, XRCC3 was present in the pre-purified material and flow-through material as expected but was absent in any of the purified fractions containing Rad51C. The addition of ADP had no effect on the co-purification, and the resulting Western analysis showed patterns very similar to those from wild-type and K113A mutant purification. All of the experiments were conducted three times from separate transformants and induced cultures, and the same results were observed.

DISCUSSION

XRCC3 is an essential protein for chromosome stability and cellular resistance to IR and certain chemical agents (1, 2, 9, 13). Despite its presumed importance in the repair of double-strand breaks through HRR, little is known regarding its biochemical properties or specific function. Although it has been shown that XRCC3 directly interacts with Rad51C to form a stable complex, the biological significance of this complex formation remains to be defined. We hypothesized that Rad51C-XRCC3 complex formation is necessary for the biological function of XRCC3, as measured by complementation of the MMC-sensitive mutant cell line irs1SF. However, we found that this complex can still form with the K113A mutation, which impairs HRR capacity. We showed that the biological activity of XRCC3 depends on its ability to bind and hydrolyze ATP, as neither K113R nor K113A mutants were able to complement the defect in irs1SF. Our wild-type transfectant clones showed partial resistance to MMC and IR. Because the sensitivity of irs1SF to MMC is much greater (75-fold) than that for IR (2-fold), we used MMC resistance as the main end point for measuring biological activity by complementation of colony-forming ability. Clone 1SFK8 shows better correction for MMC and IR

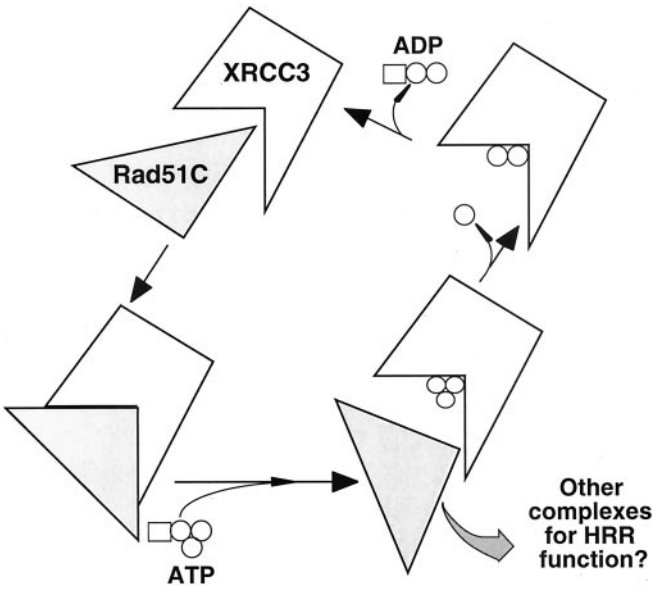

FIG. 5. **Heuristic model of the role of ATP binding in disrupting Rad51C-XRCC3 complex formation.** The Rad51C-XRCC3 heterodimer identified *in vivo* by co-immunoprecipitation is regulated by ATP binding, which dissociates the complex. Upon ATP hydrolysis and release of bound ADP, XRCC3 can bind free Rad51C. Unbound Rad51C is free to bind Rad51B and participate in the XRCC2-Rad51D-Rad51C-Rad51B complex (4).

sensitivity than previously reported clones, which makes this a valuable isogenic control for future experiments conducted on irs1SF.

Our results suggest that the ATPase activity of XRCC3 is not required for complex formation with Rad51C, since mutant K113A forms a stable complex with Rad51C. If Rad51C binds to XRCC3 in a manner analogous to how Rad51 forms its ring structure, the ATP binding pocket may be created by the Walker A box of XRCC3 and the Walker B box of Rad51C. If so, it is possible that ATP binding in this pocket would disrupt the heterodimer by pushing the two proteins apart at the binding interface. Because the K113A mutation should not allow for ATP binding, it is possible that the two proteins may join together in the absence of such steric hindrance. In support of this hypothesis that ATP binding causes steric hindrance between Rad51C and XRCC3, we did not detect wild-type XRCC3-Rad51C complex in the presence of excess ATP. The addition of ADP did not affect the binding of wild-type XRCC3 to Rad51C. Thus, although ATP binding itself inhibits complex formation, ATP hydrolysis is apparently unnecessary for complex formation. The finding that K113A transfectants (which express a XRCC3 mutant that maintains the ability to form the Rad51C-XRCC3 complex) do not show complementation for MMC resistance suggests that complex formation alone is insufficient for the function of XRCC3. We speculate that ATP hydrolysis may be required for complex dissociation, which may be required for biological activity. ATP hydrolysis activity of XRCC3 appears necessary at a step other than in the formation of a stable Rad51C-XRCC3 complex. A theoretical role for the Rad51C-XRCC3 complex could be to bring Rad51C into the nucleus, upon which the complex must dissociate via ATP hydrolysis so that Rad51C can associate with the other paralogs for it to participate in HRR. We are currently pursuing evidence for this model.

Interestingly, we found that K113R protein was insoluble in the bacterial lysate but soluble in CHO cells, suggesting that K113R may exist in a complex with a stabilizing partner other than Rad51C *in vivo*. On the other hand, K113A was stable in bacteria but appeared unstable in CHO cells, where the levels

were very low and few transformants had detectable protein. These results suggest that ATP binding may allow XRCC3 to interact with other protein partners *in vivo*.

Our results support the hypothesis that ATP binding by XRCC3 disrupts the *in vivo* Rad51C-XRCC3 complex, whereas ATP hydrolysis by XRCC3 is required at a subsequent step in HRR. Because the ring structure for Rad51 seems to be the "inactive" form of Rad51, we speculate that the Rad51C-XRCC3 complex may be an analogous "resting" state for this heterodimer (Fig. 5). Our observation that very high expression of wild-type XRCC3 gave less complementation of MMC sensitivity than lower levels may also support this model. If high overexpression of XRCC3 results in a greater proportion of Rad51C in Rad51C-XRCC3 inactive complex, perhaps leading to the disruption of the Rad51B-C-D-XRCC2 complex, HRR efficiency could be reduced.

Of the three paralogs studied for the effect of ATPase mutations on functional complementation in hamster mutant cells, XRCC3 has the most severe phenotype. A recently identified naturally occurring Walker B mutant of XRCC3 completely failed to complement the MMC sensitivity in irs1SF (27), indicating that both Walker A and B boxes of XRCC3 are required. Conservative but not nonconservative Walker A mutation in Rad51C retained the ability to partially complement mutant irs3 cells (21). Somewhat surprisingly, the nonconservative mutation in XRCC2 retained its functional activity as measured by complementation of irs1 cells (20). Even the ΔG53ΔK54 XRCC2 deletion protein was functional. If the ATPase domains of the Rad51 paralogs are structured in an analogous way to those of Rad51 heptamers, the Walker B box mutants of certain paralogs may be the more deleterious. Further experimentation is necessary to address the importance of the Walker B motifs in the paralogs besides XRCC3 and to construct a more accurate picture of complex formation and function.

Alternatively, the differential requirement for the ATPase motifs may result from nonequivalent uses of these active sites. The RuvB hexamer, the bacterial helicase that is involved in HRR and resolution of Holliday junctions, utilizes only two of its six ATP-binding sites (28). Because the Rad51 paralogs have been reported to associate with Holliday junctions (22, 29, 30), we speculate that these proteins may be functional homologs of the RuvB proteins, which utilize their ATPase binding sites in a similar manner. Further biochemical analysis is required to ascertain the exact role of ATP hydrolysis by the paralogs, but our genetic studies provide strong evidence that both ATP binding and hydrolysis by XRCC3 are necessary for HRR.

In conclusion, we show that ATP binding and hydrolysis by XRCC3 influence Rad51C-XRCC3 complex formation and directly contribute to the functions of XRCC3 in HRR. We present the novel result that XRCC3 K113A, which complexes with Rad51C *in vitro*, lacks *in vivo* function. Both ATPase activity and the ability for the complex to form and dissociate seem necessary for HRR. Our results suggest that the Rad51C-XRCC3 complex represents an inactive state. These results directly contribute to our understanding of the biochemistry of Rad51 paralogs and the roles these proteins play in HRR.

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